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The E7 Oncoprotein of Human Papillomavirus Type 16 Interacts with F-Actin *in Vitro* and *in Vivo*

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We report here that E7 oncoprotein of human papillomavirus type 16 (HPV-16) forms a complex *in vivo* and *in vitro* with actin, one of the components of the cellular cytoskeleton. The *in vivo* interaction was detected by immunofluorescent staining and confocal microscopic examination of normal human oral keratinocytes (NHOK) and CV-1 cells after transient expression of E7 employing the vaccinia virus–T7 RNA polymerase system and by coimmunoprecipitation from an immortalized, nontumorigenic cell line obtained after transfecting NHOK with the cloned HPV-16 DNA genome. The *in vitro* interaction was detected by cosedimentation of bacterially expressed E7 phosphorylated with rabbit reticulocyte lysate or purified casein kinase II (CKII) prior to incubation with F-actin. This interaction was inhibited if E7 phosphorylation by the rabbit reticulocyte lysate was prevented with heparin, a CKII inhibitor, or if the amino acids Ser-31 and Ser-32 in E7, which are phosphorylated by CKII, were replaced with amino acids that cannot be phosphorylated. Interestingly, a decrease in the amount of polymerized actin occurred in cells expressing E7.

Key Words: HPV; cytoskeleton; E7 oncoprotein; viral carcinogenesis.

INTRODUCTION

One of the hallmarks of malignant transformation is the alteration of the cellular cytoskeleton, resulting in decreased adhesion and modified adhesion-dependent responses (Ben-Ze'ev, 1997; Fey and Penman, 1985; Janmey and Chaponnier, 1995). These alterations involve structural components of the cytoskeleton or proteins that interact with this structure at different stages of the cell cycle (Janmey and Chaponnier, 1995). The major transforming proteins encoded by the "high-risk" human papillomaviruses (HPVs), E6 and E7 (Baker *et al.,* 1987; Munger and Phelps, 1993), bind and inactivate the tumor suppressor p53 and pRb proteins, respectively, causing a disruption in cell cycle control (zur Hausen and de Villiers, 1994). These oncoproteins also interact with several other cellular proteins, e.g., cyclin A, AP-1, E2F1, $p21^{WAF1/CIP1}$, and $p27^{kip1}$ (Los Alamos National laboratory Web site, 1997), which may, in part, be responsible for the oncogenicity of these viruses (zur Hausen and de Villiers, 1994).

Interaction between the cytoskeleton and viral proteins has been implicated in the replication and maturation of several human viral pathogens, including vaccinia virus, human immunodeficiency type 1 virus, measles virus, and human respiratory syncytial virus (Bohn *et al.,* 1986; Burke *et al.,* 1998; Cudmore *et al.,* 1995; Rey *et al.,* 1996). Modifications of the cytoskeleton as a result of viral protein expression have been associated with oncogenic transformation by Epstein–Barr virus, Rous sarcoma virus, and papillomaviruses (Felice *et al.,* 1990; Martin and Sugden, 1991; McCormack *et al.,* 1997; Tong and Howley, 1997; Tong *et al.,* 1997). By analyzing the intracellular distribution of HPV-16 E7 during transient expression of the viral oncogene, we found that a fraction of this protein colocalized with F-actin. Similar analysis of a human oral keratinocytes immortalized with cloned HPV-16 genome also showed an interaction between E7 and actin. We also demonstrated this association *in vitro* by cosedimentation of F-actin and E7. Interestingly, the *in vitro* interaction was abolished when the CKII activity, which is involved in E7 phosphorylation (Firzlaff *et al.,* 1989), was inhibited by heparin treatment. Furthermore, if the E7 amino acids that are phosphorylated by CKII were replaced with amino acids that cannot be phosphorylated, the cosedimentation of E7 and F-actin was also abolished. Interestingly, a decrease in the amount of F-actin was detected in cells expressing the HPV-16 E7 protein.

RESULTS AND DISCUSSION

Intracellular distribution of E7

The intracellular distribution of E7 was analyzed after transient expression of this protein in primary normal

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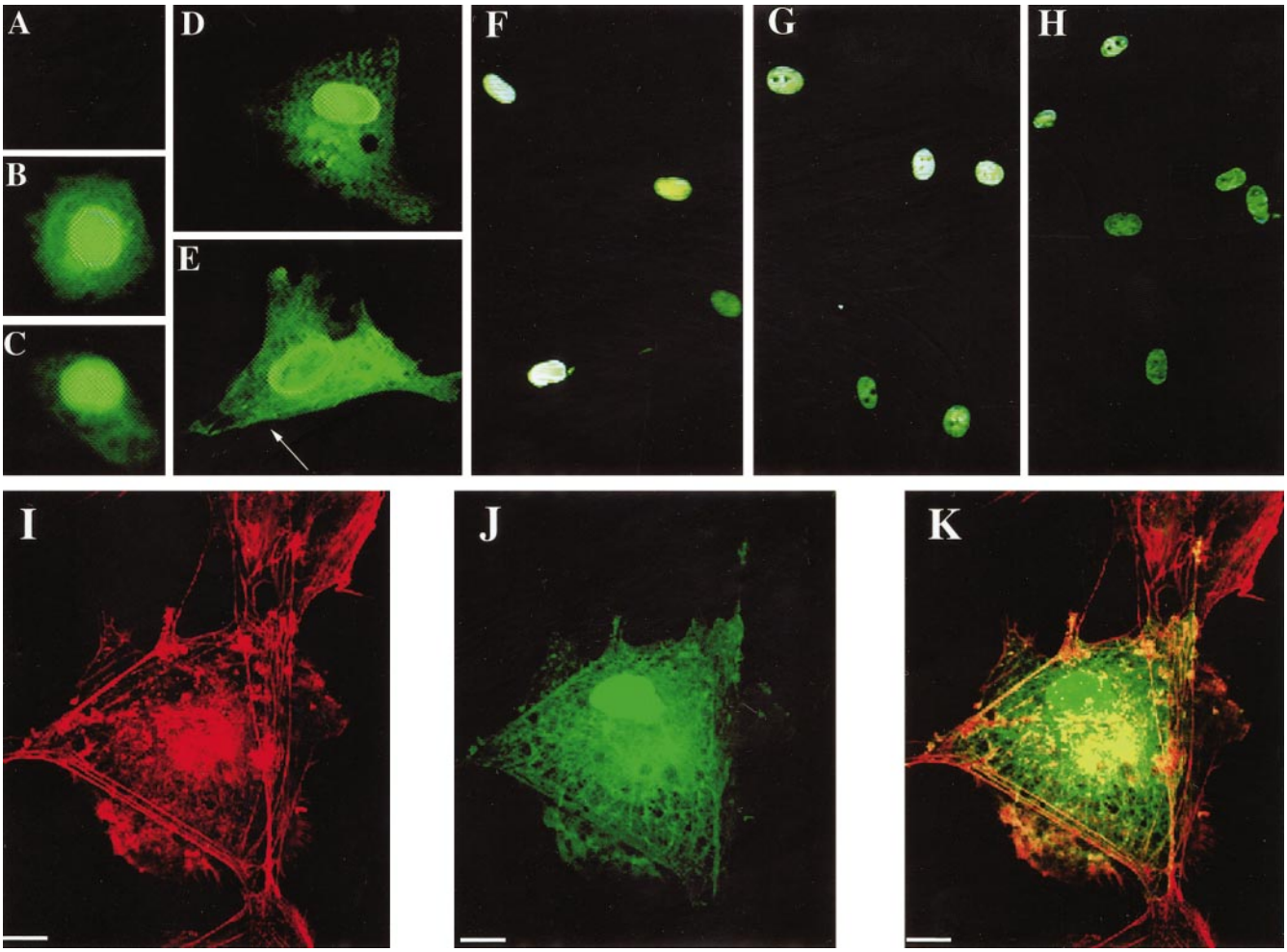

FIG. 1. Intracellular E7 protein distribution. NHOK (A–C) or CV-1 cells (D–J) infected with vTF7-3 (A–F and H–J) and transfected with pGem4Z (A and H), pGE7 (B–E, I, and J) or pGNP (F) were fixed 3 h posttransfection and processed for indirect immunofluorescence (A–H) or confocal microscopy (I and J). E7 was detected using a murine monoclonal antibody and fluorescein-conjugated goat anti-mouse immunoglobulins (A–E and J). Influenza NP was detected using a pool of two murine moclonal antibodies and fluorescein-conjugated goat anti-mouse immunoglobulins (F). $p16^{INK4a}$ was detected using a murine monoclonal antibody and fluorescein-conjugated goat anti-mouse immunoglobulins (G, H). For actin detection, the specimen was costained with Texas red-conjugated phalloidin (I) after E7 immunostaining. (K) The superimposed images of I and J. (A–E) ×1000 magnification; (F–H) ×200 magnification; bars in I–K represent 10 μm.

human oral keratinocytes (NHOK) using the vaccinia virus–T7 RNA polymerase system. Cells infected with a recombinant vaccinia virus encoding T7 RNA polymerase (vTF7-3) were transfected with a vector encoding HPV-16 E7 under the control of the T7 polymerase promoter (pGE7) and processed for indirect immunofluorescence 3 h later. A low multiplicity of infection (m.o.i.) (5 PFU/cell) and short time postinfection (less than 4 h) were employed in our experiments to minimize the cytopathic effects (CPE) associated with vaccinia replication. As previously reported, vaccinia caused CPE at early times postinfection only when a high m.o.i. (>150 PFU/cell) was employed (Bablanian *et al.,* 1978; Bablanian, 1975). NHOK were stained using a murine monoclonal antibody against E7 (MAbE7) as primary antibody and fluorescein-conjugated goat anti-mouse immunoglobulin as secondary antibody. There was no staining in control cells infected with vTF7-3 and transfected with empty vector (pGem4Z) (Fig. 1A). In contrast, approximately 5% of cells transfected with pGE7 showed a strong nuclear signal and a weaker cytoplasmic signal (Figs. 1B and 1C). The remaining cells (~95%) were unstained and similar to the control. We used CV-1 cells for the remaining experiments because the transfection rates in CV-1 were higher than in NHOK. The transfection rate in CV-1 cells was close to 15%. These cells showed a distribution of E7 similar to that observed in pGE7 transfected NHOK (Figs. 1D and 1E). Furthermore, CV-1 cells infected with vTF7-3 and transfected with the empty vector pGem4Z were not stained by MabE7 (data not shown). Examination of the E7 distribution in CV-1 cells revealed a fiber-like staining pattern within the cytoplasm (Fig. 1E, arrow). Similar results were obtained employing another murine monoclonal antibody against E7 (TVG710Y) (data not

shown). In order to rule out nuclear leakage due to CPE caused by vaccinia virus, we tested under similar conditions the distribution pattern of the influenza virus nucleoprotein (NP), which localizes into the nucleus (Davey *et al.,* 1985). When NP (56 kDa) was expressed using the vaccinia/T7 polymerase system, it was detected in the nucleus as previously reported (Fig. 1F). In addition we studied the intracellular distribution of an endogenous cellular protein, the tumor suppressor $p16^{INK4a}$ (16 kDa) (Serrano, 1997), in vaccinia-infected CV-1 cells. We did not find any difference in the distribution of this protein when compared to mock-infected CV-1 cells (Figs. 1G and 1H). Although high-risk HPVs E7 protein is predominantly nuclear, several reports have indicated that it can also be detected in the cytoplasm of eukaryotic cells, albeit at a much lower level than in the nuclei (Bernard *et al.,* 1987; Kanda *et al.,* 1991). These authors found that a fraction of E7 could be detected in the cytoplasm regardless of different levels of E7 expression or the use of different cell lines. Since none of these investigators employed vaccinia virus in their expression systems, it is very unlikely that the E7 protein they detected in the cytoplasm resulted from nuclear leakage. Furthermore, indirect immunofluorescence revealed E7 to be associated with fiber-like structures in the cytoplasm of COS-1 cells (Kanda *et al.,* 1991).

CV-1 cells infected with vTF7-3 and transfected with pGE7 were costained with MAbE7 and fluorescein-conjugated goat anti-mouse immunoglobulin followed by Texas red-conjugated phalloidin and analyzed by confocal microscopy to reveal F-actin and E7 distribution, respectively (Figs. 1I and 1J). Many prominent microfilaments running through the cytoplasm of CV-1 as well as several short microfilaments and speckled staining revealed the F-actin distribution pattern in the cytoplasm of these cells (Fig. 1I). The longest microfilaments were located mainly near the cell periphery, with some of them making contact between two neighboring cells that showed a similar F-actin pattern (Fig. 1I). In CV-1 cells expressing E7, immunostaining and confocal microscopic observations revealed E7 localization, which was very similar to that described in NHOK cells (Fig. 1J). Although the strongest E7 signal was detected in the nucleus, several discrete fibers at the cell periphery also reacted with the anti-E7 antibody. When both images were superimposed to compare the distribution of E7 and F-actin, they revealed that a fraction of E7 colocalized with peripheral microfilaments (Fig. 1K). A diffuse signal of E7 apparently not associated with F-actin was also visible in this superimposed image. Aggregates in the cytosol, which were stained by Texas red-conjugated phalloidin, also reacted with the anti-E7 antibody and they colocalized in the superimposed image. The origin of these structures is currently unknown. However, there was no difference in the microfilaments of mock-infected CV-1 cells compared to CV-1 cells infected with vaccinia virus and transfected with the empty vector under our experimental conditions (data not shown).

No bleed-over was observed between the signals from fluorescein and Texas red stains between filter settings. This phenomenon was demonstrated by two cells that showed only F-actin and were in close contact with the cell (the cell in the center of the figure) showing both E7 and F-actin, as well as by the lack of signal in the red channel from the strong E7 nuclear staining (compare Figs. 1I and 1J).

Similar results were obtained employing a different murine monoclonal antibody to E7(TVG710Y) (data not shown). The colocalization of transiently expressed E7 and F-actin was observed as early as 2 h posttransfection (data not shown). This observation suggested that the detected colocalization was not an artifact caused by overexpression of E7, since the level of E7 present at that time was considerably lower than that at later times.

In vivo interaction of E7 and actin

The interaction of E7 with cellular microfilaments noted above could have resulted from the relatively high level of E7 expression attained with the vaccinia expression system. These could cause a nonspecific interaction between microfilaments and E7. To address this question and confirm the *in vivo* interaction of E7 and actin in a system where E7 expression was more "physiological," we utilized a cell line (HOK-16B) that expressed a low level of E7. HOK-16B cells were established by transfection of primary NHOK with the cloned HPV-16 genome (Park *et al.,* 1991). HOK-16B cells not only expressed very low levels of E7, e.g., 5.5-fold less than CaSki cells (Ke *et al.,* 1999), but also had the following advantages: (1) they are immortalized nontumorigenic cells; (2) their origin from NHOK has been clearly established; and (3) an appropriate experimental control (NHOK cells) is readily available. Because we could not detect the E7 protein by immunofluorescence, very likely due to its low level (Ke *et al.,* 1999), analysis of the interaction between E7 and actin was conducted in HOK-16B cells by radioimmunoprecipitation and Western blot analysis.

NHOK and HOK-16B cells were pulse-labeled and enucleated cell lysates immunoprecipitated under nondenaturing conditions with antibodies against actin or E7. The immunocomplexes were resolved by SDS–PAGE and transferred to a membrane. The top portion of the membrane, between the 30- and the 66-kDa markers, was excised and incubated with an anti-actin murine monoclonal antibody and alkaline phosphatase-conjugated goat anti-mouse antibody. Signals were detected employing a chemifluorescent substrate and a phosphorimager (Fig. 2A). As indicated under Materials and Methods, since actin has a very slow turnover, which made its detection by radiolabeling very inefficient (Chiu

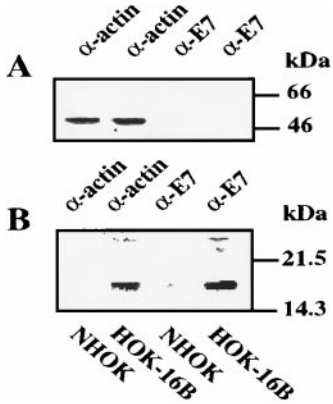

FIG. 2. Coimmunoprecipitation of E7 and actin. NHOK or HOK-16B cells were pulse-labeled and lysed, and the proteins were immunoprecipitated under nondenaturing conditions using anti-actin (α-actin) or anti-E7 (α-E7) murine monoclonal antibodies. The proteins resolved by SDS–PAGE were transferred to a membrane using standard procedures. The top portion of the membrane was incubated with α-actin as primary antibody and alkaline phosphatase-conjugated anti-mouse as secondary antibody for actin detection (A). Signals were detected with a chemifluorescent substrate and a phosphorimager. The lower portion of the same membrane was exposed to phosphorimager screens and signals detected with a phosphorimager (B). The position of protein molecular weight markers is indicated on the right.

and Goldman, 1984; Kreis *et al.,* 1979; Rubinstein *et al.,* 1976), its visualization was achieved by Western blot. The lower portion of the same membrane was exposed to a phosphorimager screen and signals were detected with a phosphorimager (Fig. 2B), since the small amount of E7 present in HOK-16B did not permit its detection by Western blot. A peptide migrating with an apparent size of approximately 19 kDa, corresponding to E7, was immunoprecipitated from HOK-16B but not from NHOK by anti-E7. A peptide with the same mobility was also coimmunoprecipitated by anti-actin antibodies from HOK-16B cells but not from NHOK. However, actin was not detected in the immunocomplexes formed in HOK-16B cells by using a mixture of two different anti-E7 monoclonal antibodies under the same experimental conditions. Although we do not currently have an explanation for this observation, unfavorable steric interactions resulting from the binding of antibodies to E7 may be responsible for this phenomenon. Similar results were obtained in CV-1 or NHOK cells using the transient expression system described above (data not shown). These data indicate that the association of E7 with F-actin present in microfilaments was highly specific for E7 and apparently not dependent upon the intracellular concentration of E7. Overexposure of the membrane probed with anti-actin antibodies revealed very faint bands close to the 46- and 66-kDa marker that did not comigrate with actin (data not shown). These bands may correspond to the heavy chain of anti-actin IgM (78 kDa) and anti-E7 IgG (55 kDa).

E7 can form a complex with F-actin *in vitro*

To further ascertain the E7/F-actin interaction, we employed an *in vitro* binding assay (Matsudaira, 1992; Rey *et al.,* 1996) using bacterially expressed E7 and *in vitro* polymerized actin (F-actin). This assay was based on the fact that proteins with moderate to high binding affinities ($<$10 μM kDa) for actin can be detected by cosedimentation since F-actin pellets at high (100,000 *g*) but not low (10,000 *g*) centrifugal forces (Matsudaira, 1992). Bacterially expressed glutathione *S*-transferase (GST)–E7 fusion protein was purified and the GST moiety removed by enzymatic cleavage with thrombin. The obtained E7 protein incubated with *in vitro* polymerized actin was centrifuged and equal volumes of the solubilized pellet and supernatant were analyzed by Western blot. Signals were detected as described under Materials and Methods. Unexpectedly, E7 did not cosediment with F-actin (data not shown).

This disparity between the *in vivo* and the *in vitro* experimental results could be due to the absence of a cellular factor(s) in the *in vitro* assay that is necessary for the E7 and F-actin interaction. For example, in our *in vitro* assay we used highly purified monomeric actin ($>$99%) in order to obtain F-actin. Because microfilaments in the cell are formed by polymerized actin and several other proteins, such as troponin, myosin, caldesmon, fimbrin, and adducin (Kreis and Vale, 1993), the lack of any of these proteins or other cobinding factor(s) could have affected the ability of E7 to interact with F-actin. Also, posttranslational modifications of E7, which may occur in eukaryotic cells, could be absent in the bacterially expressed protein, affecting its ability to interact with other proteins. To address these possibilities, we conducted the *in vitro* binding assay in the presence of 10 μl of rabbit reticulocyte lysate (RL), which contains a variety of posttranslational processing activities, as well as multiple cellular factors (Jackson and Hunt, 1983). As positive and negative controls, we included purified bacterially expressed HIV-1 Gag protein, which interacts with F-actin (Rey *et al.,* 1996), and GST, which does not. Under these experimental conditions, significant cosedimentation of E7 occurred with F-actin (Fig. 3). Quantitation of the obtained signals indicated that in the absence of F-actin, 10% of GST, 24% of Gag and 10% of E7 were present in the pellet. The inclusion of F-actin increased the amount of GST in the pellet by only 8%. The amount of Gag, the positive control cosedimenting with F-actin, was 34% higher than in the absence of F-actin and E7 showed a net increase of 28%. We cannot rule out the possibility that some of the E7 protein that cosedimented with F-actin resulted from trapping. Nevertheless, the results obtained with the negative controls GST and bovine serum albumin (data not shown) indicated that a significant amount of E7 cosedimented with F-actin.

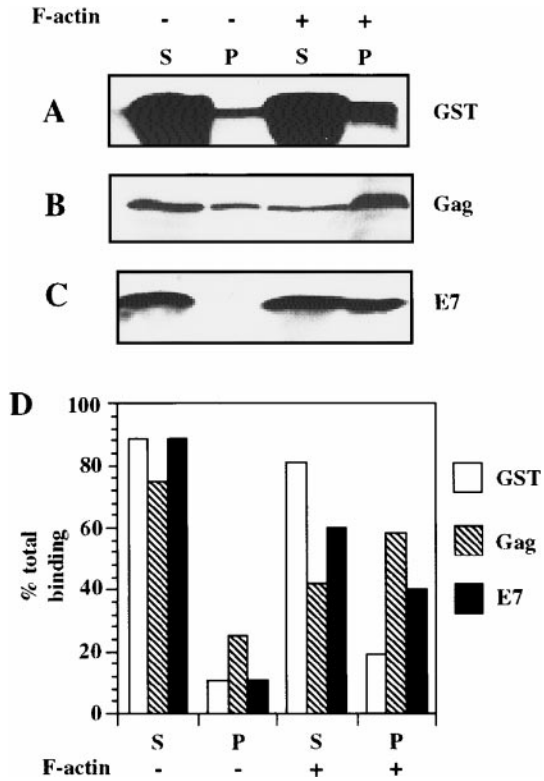

FIG. 3. E7/F-actin *in vitro* interaction. Bacterially expressed GST, HIV-1 Gag, and E7 were incubated with (+) or without (−) *in vitro* polymerized actin (F-actin) and centrifuged in a Beckman airfuge for 20 min at 100,000 *g*. The supernatant (S) and pellet (P) were analyzed by SDS–PAGE and Western blotting. GST (A), Gag (B), and E7 (C) were detected employing primary antibodies against these proteins and secondary alkaline phosphatase-conjugated antibodies. Signals were quantified using a phosphorimager and expressed (D) as a percentage of the total protein (100% equals S + P).

In vitro interaction of E7 and F-actin requires phosphorylation of E7

Previous reports have indicated that E7 can be phosphorylated by CKII (Barbosa *et al.,* 1990; Firzlaff *et al.,* 1989), a ubiquitous active kinase in the cytosol and nucleus (Edelman *et al.,* 1987; Filhol *et al.,* 1990; Hathaway and Traugh, 1982; Kandror *et al.,* 1989; Singh and Huang, 1985) that can be activated by different growth factors (Meisner and Czech, 1991). To determine whether E7 phosphorylation is mediated by CKII in the rabbit RL, heparin was included in the assay. Heparin is a potent inhibitor of CKII, which has no effect on other kinases such as casein kinase I, type I and II cAMP-dependent protein kinases, protease-activated kinase I, and the hemin-controlled repressor (Feige *et al.,* 1980; Hathaway *et al.,* 1980; Hathaway and Traugh, 1982; Maenpaa, 1977). After incubation with or without heparin, E7 was immunoprecipitated with the MAbE7, samples were analyzed by SDS–PAGE, and signals were detected with a phosphorimager. As shown in Fig. 4, the inclusion of heparin prevented the phosphorylation of E7. If phosphorylation

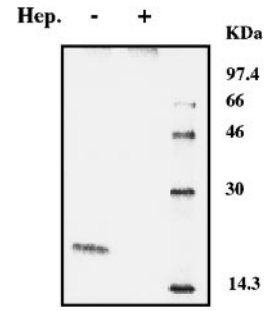

FIG. 4. *In vitro* phosphorylation of E7 protein. Bacterially expressed E7 was incubated with rabbit reticulocyte lysate (RL) and [γ-^{32}P]ATP in the presence (+) or in the absence (−) of the CKII inhibitor heparin. Samples, immunoprecipitated using a murine monoclonal antibody against E7, were resolved by SDS–PAGE. Signals were detected using a phosphorimager. Hep, heparin. The position of protein molecular weight markers is indicated on the right.

of E7 by CKII was necessary for the interaction between F-actin and this protein, prevention of E7 phosphorylation should abrogate this interaction. Indeed, when heparin was included in the *in vitro* binding assay in the presence of RL before the addition of E7, E7 did not cosediment with F-actin (Fig. 5). Moreover, the distribution of E7

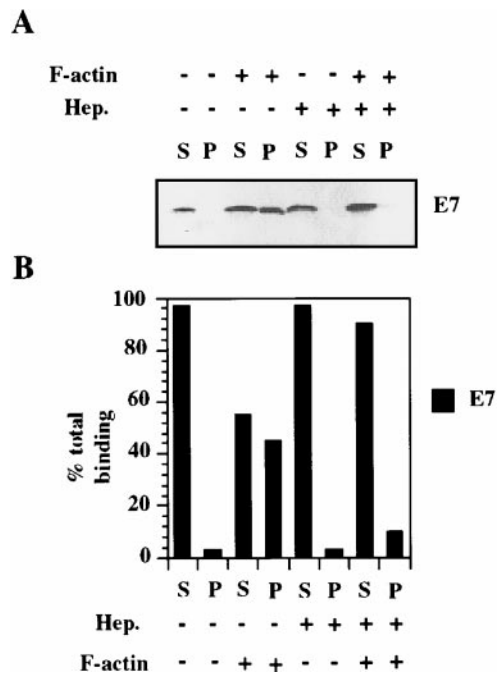

FIG. 5. Effect of CKII inhibition on E7/F-actin *in vitro* interaction. Bacterially expressed E7 was incubated with (+) or without (−) the CKII inhibitor heparin and *in vitro* polymerized actin (F-actin). The supernatant (S) and pellet (P), obtained after centrifugation of samples in a Beckman airfuge for 20 min at 100,000 *g,* were analyzed by SDS–PAGE and Western blotting. E7 (A) was detected employing a primary antibody against E7 and secondary alkaline phosphatase conjugated antibodies. Signals were quantified using a phosphorimager and expressed (B) as a percentage of the total protein (100% equals S + P). Hep, heparin.

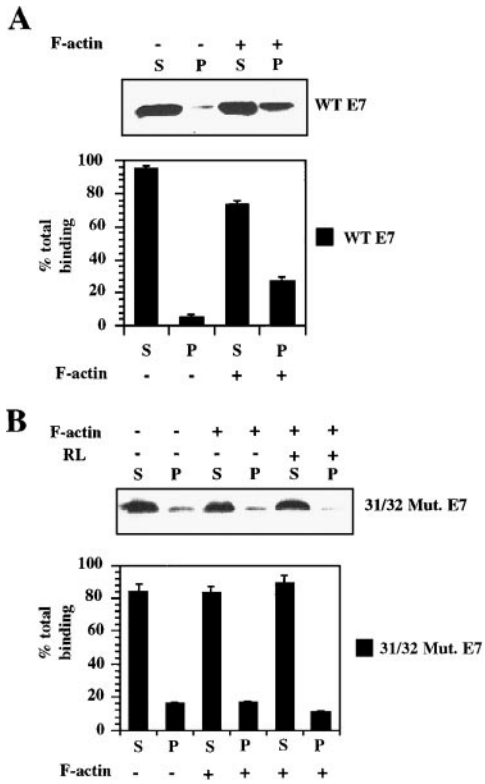

FIG. 6. E7 CKII phosphorylation-deficient mutant and F-actin *in vitro* interaction. Bacterially expressed wt E7 (A) or 31/32 mutant E7 (B) proteins were incubated with purified CKII and then with *in vitro* polymerized actin (F-actin). The supernatant (S) and pellet (P) obtained after centrifugation of the samples in a Beckman airfuge for 20 min at 100,000 *g,* were analyzed by SDS–PAGE and Western blotting. E7 was detected employing a primary antibody against E7 and secondary alkaline phosphatase-conjugated antibodies. Signals were quantified using a phosphorimager and expressed as a percentage of the total protein (100% equals to S + P). Results were the mean of three independent experiments. Bars represent the standard deviation. RL, rabbit reticulocyte lysate.

under these conditions was the same as that of E7 incubated without F-actin. When heparin was added 1 h after E7, it did not affect the ability of this protein to interact with F-actin (data not shown). These results suggest that E7 phosphorylation by CKII is required for its interaction with F-actin.

To further demonstrate that phosphorylation was needed for E7 to associate with F-actin, purified CKII was used to phosphorylate bacterially expressed E7. In addition, the 31/32 E7 mutant protein in which the amino acids Ser-31 and Ser-32, which are phosphorylated by CKII, were mutated to amino acids Ala and Ala (that cannot be phosphorylated), respectively, was constructed and tested for its ability to interact with F-actin. As shown in Fig. 6A, E7 phosphorylated by purified CKII was able to cosediment with F-actin in the absence of RL. Under these conditions the binding ability of E7 was decreased when compared to the previous results (see Figs. 4 and 5), raising the possibility that other factors present in the RL increased its binding efficiency. There was no significant difference in E7 phosphorylation by purified CKII or RL (data not shown).

The 31/32 E7 mutant protein showed, on average, a 5% net increase in its ability to pellet in the absence of F-actin when compared to the E7 wild-type (wt) protein under the same conditions (Fig. 6B). When F-actin was included in the *in vitro* binding assay and the E7 mutant protein treated with CKII, the amount of mutant E7 cosedimenting with F-actin was the same as in the absence of F-actin, indicating that the interaction between these two proteins was disrupted (Fig. 6B). The same results were obtained when RL was included in the assay with the mutant E7 protein and F-actin. Similar results were obtained with another E7 mutant protein where the 31/32 Ser/Ser residues were mutated to 31/32 Arg/Pro (data not shown).

F-actin distribution is affected in cells expressing E7

Our results indicated that the E7 protein was able to interact *in vitro* and *in vivo* with F-actin, a phenomenon that could affect the actin cytoskeleton. To address this possibility, the microfilaments of NHOK and HOK-16B cells were labeled with Texas red-conjugated phalloidin and analyzed by confocal microscopy (Fig. 7). As evident from representative images, NHOK cells had an elaborate cytosolic network of F-actin fibers (Fig. 7A) that was mostly absent in the HOK-16B cells (Fig. 7B). Although both cell types showed microspikes, these structures were more noticeable, uniform, and evenly distributed in NHOK cells. Analysis of F-actin distribution in another NHOK-derived cell line that expressed only the E7 protein (HOK-16E7) (Fig. 7C) showed the same profile as in HOK-16B, suggesting that the observed decrease in F-actin was linked to E7 expression. The difference in the actin network of NHOK with that of HOK-16B and HOK-16E7 was not caused by cell clustering or piling up because these artifacts were not observed in serial confocal images of NHOK cells (data not shown).

Under normal physiological conditions there is equilibrium between nonpolymerized, monomeric actin and polymerized actin in the cellular cytosol, with approximately half of the actin present in the nonpolymerized form (Bershadsky and Vasiliev, 1988). Since phalloidin binds only to F-actin and not to monomeric actin, the difference in immunofluorescence intensity detected between NHOK and the other two cell lines that express E7 may only reflect a shift in the distribution of actin. To determine whether there was a net difference in actin content between NHOK, HOK-16B, and HOK-16E7, cell lysates were analyzed by Western blot using an anti-actin monoclonal antibody. No significant difference was detected in the amount of actin present in HOK-16B or HOK-16E7 cells compared to NHOK cells (Fig. 8), suggesting that the decrease in F-actin detected in E7 ex-

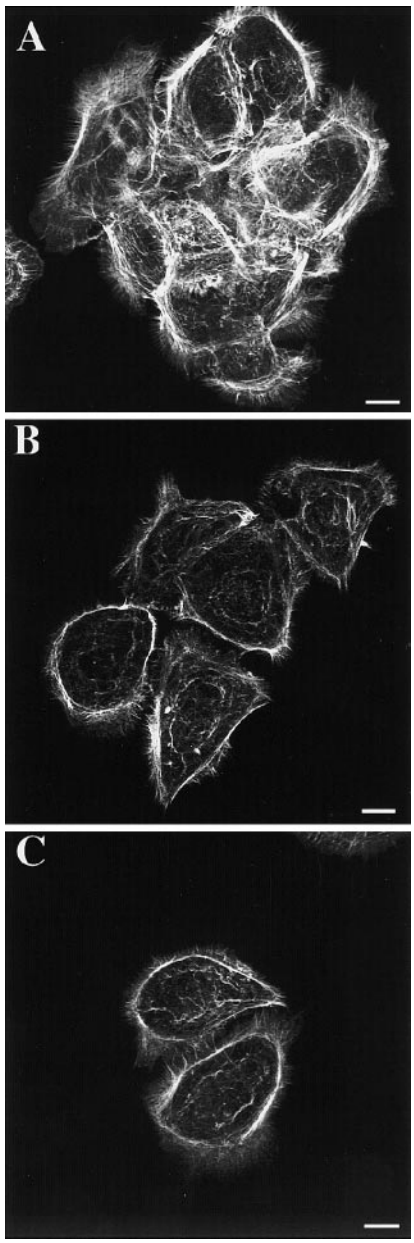

FIG. 7. Microfilament organization in cells expressing E7. NHOK (A), HOK-16B (B), or HOK-16E7 cells (C) were fixed and F-actin was detected by staining with Texas red-conjugated phalloidin and confocal microscopy. Bars represent 10 μm.

pressing cells reflects a defect in actin polymerization. The observed difference in actin distribution might be a secondary phenomenon due to immortalization in both E7 expressing cell lines and requires further analysis.

The data presented here reveal a novel function of high-risk HPV in NHOK and CV-1 cells, e.g., the association of the HPV-16 E7 oncoprotein with actin, a component of the cytoskeleton. This observation is based on results obtained from several independent *in vivo* and *in vitro* experimental approaches. Interestingly, we also noted that the actin cytoskeleton was significantly disrupted in cells expressing E7.

F-actin is part of cellular structures, e.g., microfilaments and the cell cortex, which interact with several structural and regulatory components (Schliwa, 1986). These interactions are critical for cellular morphology, cytoskeletal organization, motility, adhesion, and signal transduction (Assoian and Zhu, 1997). Therefore, F-actin modifications will not only affect cytoskeletal organization but can also disrupt several cellular functions (Ben-Ze'ev, 1997; Janmey and Chaponnier, 1995).

Modifications of the cytoskeleton resulting from viral protein expression have been implicated in the oncogenic transformation associated with several other viruses (Felice *et al.,* 1990; Martin and Sugden, 1991; McCormack *et al.,* 1997; Tong and Howley, 1997; Tong *et al.,* 1997). In the case of bovine papillomavirus type 1 (BPV-1), evidence has been presented that the E6 oncoprotein interacts with paxillin, a component of the focal adhesion. This interaction appears to be involved in the ability of BPV-1 E6 protein to disrupt the cytoskeleton as part of the oncogenic potential of this virus (Tong and Howley, 1997; Tong *et al.,* 1997). Other viruses such as measles virus, rabies virus, human respiratory syncytial virus, and HIV-1 also encode proteins that interact with the cytoskeleton; however, the role of these interactions in viral replication or their effect on their respective hosts remains speculative (Bohn *et al.,* 1986; Burke *et al.,* 1998; Rey *et al.,* 1996; Sagara *et al.,* 1995).

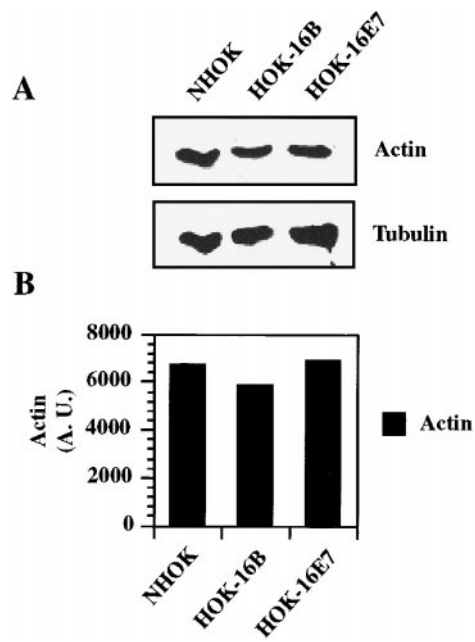

FIG. 8. Actin steady state in cells expressing E7. Total protein extracts from NHOK, HOK-16B, and HOK-16E7 were electrophoresed on SDS–10% PAGE and transferred to membranes. The membrane was sequentially decorated with murine monoclonal antibodies against actin and then β-tubulin and alkaline phosphatase-conjugated second antibody plus a chemifluorescent substrate (A). Detection and quantitation (B) were performed with a phosphorimager. The obtained actin values were normalized by comparison with the β-tubulin content. A.U., arbitrary units.

In conclusion, HPV-16 may not only inactivate the tumor suppressor proteins p53 and pRB with its E6 and E7 oncoproteins, respectively, but could also alter other cellular functions through E7 interaction with the cytoskeleton or associated proteins. This possibility is being investigated in our laboratory.

MATERIALS AND METHODS

Cells and viruses

Primary NHOK, HOK-16B cells, and NHOK expressing only HPV-16 E7 were cultured as described previously (Liu *et al.,* 1997; Park *et al.,* 1991) in keratinocyte growth medium supplemented with reagents supplied in the bullet kit (Clonetics Corp., San Diego, CA). African green monkey kidney (CV-1) cells were obtained from the American Type Culture Collection (ATCC) and maintained as recommended by the ATCC. Vaccinia virus (vTF7-3) was propagated and purified as previously described (Fuerst *et al.,* 1987).

Plasmid constructions

The plasmid pGE7, encoding the wt HPV-16 E7 protein under the control of the T7 RNA polymerase promoter, was constructed by subcloning a cDNA fragment containing the complete E7 ORF (nucleotides 505 to 1176) isolated from pE7Mo (Edmonds and Vousden, 1989) into pGem4Z (Promega Corp., Madison, WI). The construct generated was confirmed by DNA sequence analyses. The plasmid pGNP, encoding the influenza virus (A/PR/8/34 strain) nucleoprotein under the control of the T7 promoter, was a gift from D. Nayak (Biswas and Nayak, 1996).

Transient E7 protein expression

WT E7 protein expression was induced in cells using the vaccinia virus–T7 RNA polymerase system (Fuerst *et al.,* 1987). Cells were incubated for 1 h at 37°C with the recombinant vaccinia virus vTF7-3, encoding the T7 RNA polymerase, at a multiplicity of infection of 5 PFU/cell, and then transfected with pGE7 employing LipofectAMINE PLUS (Gibco BRL, Gaithersburg, MD) following the manufacturer's suggested conditions. Unless indicated otherwise, the cultures were incubated for an additional 3 h at 37°C before being processed.

Immunofluorescence and confocal microscopy

Cells cultured on Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) were fixed for 15 min at 25°C in 10% buffered formalin phosphate and permeabilized with 0.4% Triton X-100 in phosphate-buffered saline for 5 min at 25°C (Lane and Harlow, 1988). Immunostaining was performed as previously described (Rey and Nayak, 1992). F-actin was stained with Texas red-conjugated phalloidin (Molecular Probes, Eugene, OR) as recommended by the manufacturer. Imaging was performed using an Olympus BH-2 RFCA (immunofluorescence) or a C. Zeiss LSM 310 confocal microscope.

Radiolabeling, immunoprecipitation, and Western blot analyses

Coimmunoprecipitation experiments were performed with cultured NHOK or HOK-16B monolayer cells (2–3 × 10^6 cells) incubated for 30 min in cysteine-free minimal essential medium with 5% dialyzed fetal bovine serum and then pulse-labeled with 50 μCi/ml of [^{35}S]cysteine (ICN Biomedicals, Inc., Irvine, CA) for 2 h. After being labeled, the cells were lysed by incubation for 30 min at 4°C in 1 ml of 1XRIPA minus sodium dodecyl sulfate (SDS) [50 mM Tris–HCl, pH 7.6; 150 mM NaCl; 1% Triton X-100; 0.5% sodium deoxycholate; and a protease inhibitor cocktail (Boehringer Mannheim, GmbH, Germany)]. The cell lysates were centrifuged at 8000 *g* for 10 min at 4°C. Pellets containing debris, unsolubilized proteins, and nuclei were discarded. Proteins were immunoprecipitated from the supernatant at 4°C under nondenaturing conditions employing a murine monoclonal anti-actin antibody (400 ng IgM) and a mixture of two murine monoclonal anti-E7 antibodies (300 ng IgG). The immune complexes, collected with a mixture of Protein A–Sepharose (Pharmacia Biotech AB, Uppsala, Sweden) and Protein G Plus–agarose (Calbiochem–Novabiochem Corp., La Jolla, CA), were washed three times with ice-cold washing solution (50 mM Tris–HCl, pH 7.6; 500 mM NaCl; 1% Triton X-100; 0.5% sodium deoxycholate; 1% BSA; and protease inhibitor cocktail) and once with 1XRIPA minus SDS. Proteins were extracted for 10 min at 95°C in 2× SDS–sample buffer (1× SDS–sample buffer: 62.5 mM Tris–HCl, pH 6.8; 2% SDS; 2 mM EDTA; 5% 2-mercaptoethanol; 10% glycerol; and 0.001% bromophenol blue), electrophoretically separated in SDS–12% polyacrylamide gels, and transferred to Immuno-Blot PVDF membranes (Bio-Rad Laboratories, Hercules, CA) using standard procedures. Actin was detected by specific interaction of the transferred proteins in the membrane with an antibody against actin in place of exposing it to phosphorimager screens. This was necessary since actin has a very slow turnover (Chiu and Goldman, 1984; Kreis *et al.,* 1979), which made its detection by radiolabeling very inefficient. Thus, the top portion of the membranes, between the 30- and the 66-kDa markers, was excised and incubated wit primary murine monoclonal antibodies using standard procedures and alkaline phosphatase-conjugated anti-mouse secondary antibody. Signals were detected employing a chemifluorescent substrate (Vistra Systems, Amersham Life Sciences) and a Storm 840 phosphorimager. The lower portion of the same membrane was exposed to phosphorimager screens and signals detected with a Storm 840 phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Fusion protein: Construction, expression, and purification

The fusion protein plasmid encoding the wt E7 (pGEX-E7) was constructed by subcloning the blunt-ended *Nsi*I/*Nco*I fragment (nucleotides 566 to 864) isolated from pE7Mo (Edmonds and Vousden, 1989), into pGEX-4T-1 (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) that had been digested with *Sma*I and dephosphorylated. The fusion protein plasmid pGEX-E7-31/32Ala/Ala was constructed by subcloning the blunt-ended *Nsi*I/*Nco*I fragment (nucleotides 566 to 864) isolated from pGE7-31/32Ala/Ala into pGEX-4T-1 (Amersham Pharmacia Biotech, Inc.) previously digested with *Sma*I and dephosphorylated. The plasmid pGE7-31/32Ala/Ala, where the 31Ser and 32Ser amino acid residues from the E7 wt protein were mutated to 31Ala and 32Ala, was constructed by PCR site-directed mutagenesis (Ausubel, 1987) of pGE7 using the mutagenic primer 5′-GAGCAATTAATGACGCCGCAGAGGAGGAGGATGAA-3′. All the constructs generated were confirmed by DNA sequence analyses. After expression of the glutathione *S*-transferase fusion proteins in *Escherichia coli,* the proteins were purified and the GST moiety was removed by enzymatic cleavage with thrombin following standard procedures (Ausubel, 1987).

In vitro binding assay

Purified rabbit skeletal muscle actin (Cytoskeleton, Denver, CO) (4 mg/ml) was polymerized *in vitro* in a buffer containing 50 mM KCl, 1 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.4, 1 mM ATP, and 50 μg/ml phalloidin (Molecular Probes) at room temperature for 2 h (Rey *et al.,* 1996). Mutant or wt fusion proteins (6 μg) were incubated *in vitro* with polymerized actin (10 μM final concentration) in an actin binding buffer (ABB: 75 mM NaCl; 3 mM $MgCl_2$; 10 mM Tris–HCl, pH 7.4; and 3 mM ATP) at 4°C for 2 h in a final volume of 150 μl with or without rabbit RL 10 μl/reaction (Promega Corp.). Samples were then centrifuged in a Beckman airfuge for 20 min at 100,000 *g.* The supernatant was removed and the pellet resuspended in an equivalent volume of ABB. The pellet and supernatant were solubilized in 4× SDS–sample buffer and aliquots were analyzed by Western blot.

In vitro phosphorylation of E7

In vitro phosphorylation of E7 was carried out by incubating 1 μg of purified wt or mutant protein in a solution containing 75 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris–HCl (pH 7.4), and 10 μCi [γ-^{32}P]ATP (ICN Biomedicals, Inc., Irvine, CA) or 0.1 mM ATP and 5 μl of RL at 30°C for 30 min. Alternatively, the RL in the reaction was replaced by purified CK II (500 units/reaction; New England Biolabs, Inc., Beverly, MA) and 0.1 mM ATP. Heparin (10 μM final concentration; Sigma, St. Louis, MO) was used as an inhibitor of CKII activity (Hathaway and Traugh, 1982).

Antibodies

The following antibodies were used: a murine monoclonal antibody against HPV-16 E7 protein (TVG710Y; Santa Cruz Biotechnology, Santa Cruz, CA); a murine monoclonal antibody specific for $p16^{INK4a}$ (F-12; Santa Cruz Biotechnology); murine monoclonal antibodies specific for actin and for β-tubulin (Amersham Pharmacia Biotech); a murine monoclonal antibody against HPV-16 E7 (Zymed Laboratories Inc., San Francisco, CA); alkaline phosphatase-conjugated goat anti-mouse antibodies (Santa Cruz Biotechnology); fluorescein-conjugated goat anti-mouse antibodies (Organon Teknica Corp., West Chester, PA); and murine monoclonal antibodies against influenza virus NP (H16-L10-4R5 and 46/4, ATCC).

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